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Mitofusin 2 protects effect of breviscapine against hepatic ischemia/reperfusion injury: Regulation of endoplasmic reticulum stress and apoptosis

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Abstract: *To investigate the effect of breviscapine against hepatic ischemia/reperfusion (I/R) injury and its underlying mechanisms in rats, 40 Sprague-Dawley male rats were randomly assorted into five groups (n=8 per group) as follows: Sham, I/R1 + normal saline (NS), I/R1 + breviscapine (Bre), I/R2 + NS and I/R2 + Bre, where label 1 and 2 denote ischemia time for 20 and 60 min, respectively. Bre or NS (10 mg/kg) was administered to the tail vein 1 h prior to surgery and immediately postoperatively. Reperfusion for 6 h, the blood and hepatic samples were collected immediately to evaluate hepatic function and histopathological changes, in addition with the expression levels of mitofusin 2 (Mfn2) and endoplasmic reticulum stress (ERS)-associated proteins. The results demonstrated that breviscapine decreased the elevated serum level of ALT and AST and ameliorated histopathological changes compared with the NS groups ($P<0.05$). The expression of Mfn2 and Glucose-regulated protein 78 (GRP78) and Bcl2 protein were significantly increased in the Bre groups ($P<0.05$), accompanied by decreasing the expression of C/EBP homologous protein (CHOP) and cleaved caspase-3 ($P<0.05$) compared with the NS groups. These data revealed that breviscapine could ameliorate the hepatic I/R injury, possibly contribute to the up-regulation of Mfn2 expression to inhibit the ERS pathway, thus reducing the apoptosis of hepatocyte.*

Keywords: *Hepatic ischemia/reperfusion; Breviscapine; Mitofusin 2; Endoplasmic reticulum stress; Apoptosis*

1. Introduction

Hepatic ischemia/reperfusion (I/R) injury, an inevitable pathophysiological process, raises clinical concern in trauma, hemorrhagic shock, hepatic transplantation and hepatectomy, etc. Hepatic dysfunction, hepatic failure, even multiple organ dysfunction and death, which are more prone to occur in patients with hepatic steatosis or cirrhosis, affect the prognosis seriously. Apoptosis plays a crucial role as one of the main mechanisms in hepatic I/R injury. It is currently believed that there are three kinds of apoptotic pathways in cells: mitochondrial apoptotic pathway, death receptor apoptotic pathway and endoplasmic reticulum stress (ERS) pathway. Among them, the ERS pathway has attracted more and more attention in recent years. Various stress conditions can cause of endoplasmic reticulum dysfunction, resulting in unfolded or misfolded proteins melange accumulated in the endoplasmic reticulum, breaking down the intracellular homeostasis and triggering ERS [1]. Previous studies have pointed out that ERS are involved in hepatic I/R injury and excessive ERS will further mediate hepatocyte dysfunction and hepatocyte apoptosis, aggravating hepatic I/R injury [2]. Therefore, intervention of ERS may provide a new therapeutic strategy for hepatic I/R injury. Our previous studies have also demonstrated that hepatic I/R injury could be alleviated by inhibiting ERS-induced apoptosis, but its exact pathogenesis is yet known clearly [3]. Mitofusin 2 (Mfn2), a regulator of mitochondrial, is a dynamin-like protein that mainly exists in the outer membrane of the mitochondrion, while a minor portion distributed on the endoplasmic reticulum involved in mitochondrial-associated endoplasmic reticulum membrane [4,5]. Previous studies indicate that ERS state could reduce the expression of Mfn2, which cause the abnormity of morphology and function in mitochondrial and leads to cell apoptosis [6] Therefore, analyzing the differences of Mfn2

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may help to clarify the molecular mechanisms involved in hepatic I/R injury.

Breviscapine, composes of more than 95% of scutellarin as active ingredients, is a flavonoid extracted from one common Chinese medicine named Erigeron breviscapus [7] It is widespread used in clinical practice as in cardiovascular and cerebrovascular field own to its antioxidation. Accumulating evidences point out that breviscapine also has a defensive effect against I/R injury, and its mechanism may be related to the inhibition of apoptosis [8,9]. Furthermore, our previous finding had also demonstrated that breviscapine has been shown to have beneficial effects in hepatic I/R injury, suggesting that breviscapine inhibited mitochondrial apoptotic pathway and suppressed hepatocyte apoptosis [10]. Here, we hypothesize that the protective effect of breviscapine on hepatic I/R injury also involves in the role of ERS-induced apoptosis.

Therefore, the objective of the present study was designed to determine whether ERS-induced apoptosis is involved in the protective effect of breviscapine on hepatic I/R injury. To testify this hypothesis, we treated rats that underwent hepatic I/R injury after different durations of ischemia with breviscapine and investigated ERS pathway.

2. Materials and methods

2.1 Experimental animals

The animal experiments in this study were approved and performed in accordance with the guidelines of the Laboratory Animal Ethics Committee of Jinan University. Forty male Sprague-Dawley rats were purchased from Jinan Peng Yue Experimental Animal Breeding Co., Ltd (Shandong, China) in the range of 225-250g. These rats were maintained in a pathogen-free condition under a favorable circumstance with 12/12 h light/dark cycle, supported with ad libitum food and tap water.

2.2 Experimental grouping

All rats were randomly assorted into five groups (n=8 per group) as follows: Sham group: performed similar operation without vessel occlusion. I/R1 + normal saline (NS) group: administration of NS with 20 min ischemia. I/R1 + breviscapine (Bre) group: administration of Bre with 20 min ischemia. I/R2 + NS group: administration of NS with 60 min ischemia. I/R2 + Bre group: administration of Bre with 60 min ischemia.

2.3 Establishing hepatic I/R models

In the Bre treatment groups, 10 mg/kg of breviscapine (Hunan Hang Seng Pharmaceutical Co., Ltd) was injected via the tail vein 1 h prior to surgery and immediately postoperatively. While in the NS groups, all procedures were performed as aforementioned with equivalent volume of NS instead of Bre. Fasting but provided with adequate tap water 12 h prior to surgery and then anesthetize with 10% chloralhydrate (4 ml/kg, i.p.), surgery procedures were performed as previously described [11]. Firstly, expose the hilum of the liver by median laparotomy. Secondly, clamping up the portal triads of the left and median liver lobes with a non-invasive vascular clamp, establishing approximately 70% of liver ischemia model. Thirdly, removing the clamp after its pre-set ischemic time of each groups. Finally, euthanizing the rats after 6 h reperfusion, collecting the inferior vena cava blood and the left lobe of liver sample for later process immediately.

2.4 Serum transaminases levels

About 3~4 ml of inferior vena cava blood was collected and centrifuged to obtain serum for evaluation of liver function. Using an Autolab Analyzer (Rome, Italy), the contents of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assessed. The units were expressed as per liter (U/l).

2.5 Histological assessment

Slides procedure of liver sample: Fixation in 10% neutral buffered formalin, embedded in paraffin, sliced into 4 μm sections and stained with hematoxylin-eosin (HE). Histopathological examination of liver sample for liver damage assessment under light microscope. The histologic injury of each sample

ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

was analyzed by different pathologists who were blinded to the research using Suzuki's pathology score.

2.6 TUNEL assay

In accordance with the manufacturer's instructions, using an in situ cell death detection kit (Roche, Germany) to perform the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling method (TUNEL) for assessing the hepatocellular apoptosis. Selecting 5 non-overlapping serial scopes from each slide randomly, the number of TUNEL-positive cells was counted under a microscope (Nikon 80i) at x400 magnification. Calculating and averaging the apoptotic index (AI) of each slide: (apoptotic cells/total number of cells) × 100%.

2.7 Western blotting

Western blot analysis to evaluate the expression levels of Mfn2, GRP78, Bcl2, CHOP and cleaved caspase-3. Following up the manufacturer's instructions, firstly, grinding approximately 100 mg liver sample and then lysing in the lysis buffer (Promega, Madison, WI, USA), acquires the homogenized solution. Secondly, centrifuging the homogenized solution at 850 x g for 10 min at 4°C, taking the supernatants for another centrifugation at 10,000 x g for additional 10 min at 4°C, collecting the latest supernatants for Western blot analysis. Meanwhile, measuring these proteins concentration by a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Thirdly, taking a certain amounts of protein samples into the 10% SDS-polyacrylamide gels for separation, then transferred them onto the polyvinylidene difluoride (PVDF) membranes. Fourthly, blocking the membranes with 5% fat-free milk for 1 h at room temperature, then performing incubation with primary antibodies (Cell Signaling Technology, Beverly, MA, USA) (dilute at a ratio of 1:1000 at 4°C for overnight). Fifthly, using the corresponding secondary antibodies to identify the primary antibody binding (at room temperature for 1 h) after washing with tris-buffered saline solution. Finally, adding the enhanced chemiluminescence reagents (ECL; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Those bands became detectable and visualized under the FluorChem 5500 imaging system (Alpha Innotech Corp., San Leandro, CA, USA). After that, using Image J 1.50 software (National Institutes of Health, Bethesda, MD, USA) to perform densitometric analysis, then further analyze the relative intensity of those bands.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

According to the manufacturer's protocol, firstly, using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to extract all the RNA of hepatic sample, then measure the purity and concentration of these RNA. Secondly, taking 100 ng of these RAN to perform reverse-transcription with Prime Script RT Master Mix (Takara Biotechnology Co., Ltd, Dalian, China) in a 20 μl final reaction volume, complementary DNA (cDNA) were collected. The primer sequences: β-actin (forward) 5'TGCTATGTTGCCCTAGACTTCG3', (reverse) 5'GTTGGCATAGAGGTCTTTACGG3'; Mfn2 (forward)5'GATGACAGAGGAAGTGGAAAGGC3',(reverse)5'ACAGACACAGGAAGAAGGGGC T3'. Finally, using SYBR Green PCR Master Mix SYBR Premix Ex Taq™ II (Takara Co., Ltd, Dalian, China) to perform qPCR on a Mastercycler ep realplex4 (Eppendorf, Hamburg, Germany), evaluating the relative expression. Meanwhile, normalization the expression profiles of Mfn2 gene into β-actin for further calculation by means of real-time quantitative PCR and the $2^{-\Delta\Delta Cq}$.

2.9 Statistical analysis

SPSS19.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis on all experimental data. The differences of groups were analyzed by one-way analysis of variance in mean ± standard deviation (SD). A value of $P<0.05$ was considered statistically significant.

3. Results

Breviscapine treatment reduces serum transaminases levels after I/R injury. To identify the effects of breviscapine on hepatocytes, the serum levels of ALT and AST were measured after 6 h reperfusion. As shown in Fig. 1, treated with NS, the levels of serum ALT and AST under 20 min ischemia (179.75 ± 30.54 U/l and 647.88 ± 73.55 U/l, resp.) were obviously higher than that in the sham group (85.31 ± 11.24 U/l and 399.13 ± 30.38 U/l, resp.) ($P<0.001$). Moreover, when it up to the 60 min ischemia, the levels of serum ALT and AST (652.75 ± 40.12 U/l and 1009.00 ± 110.65 U/l, resp.) were much higher

ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

than that in the sham group (P<0.001). However, as it came to breviscapine treatment, the levels of ALT and AST under 20 min ischemia were significantly decreased compared with NS groups (P<0.05). The differences were more apparently when it went up to 60 min ischemia (P<0.01). These findings suggest that breviscapine protects hepatocytes from I/R injury in individual layer.

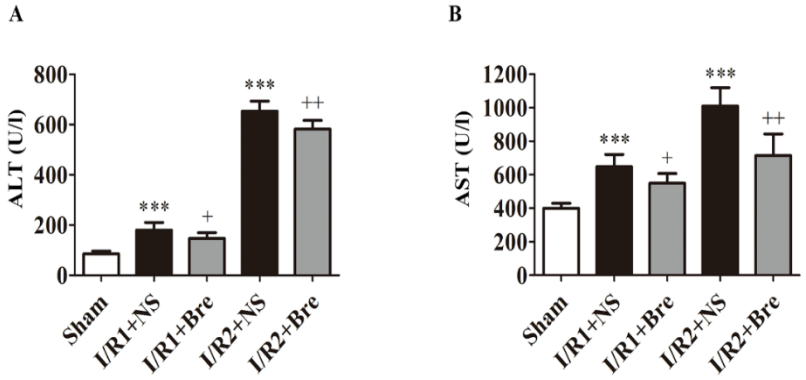

*Figure 1: Serum levels of transaminase were estimated in different groups after 6 h reperfusion. (A) Serum alanine aminotransferase (ALT) levels in different groups. (B) Serum aspartate aminotransferase (AST) levels in different groups. All data were showed in mean ± standard deviation. (n=8). ***P<0.001 vs. Sham group; $^{+}$P<0.05, $^{++}$P<0.01 vs. NS groups.*

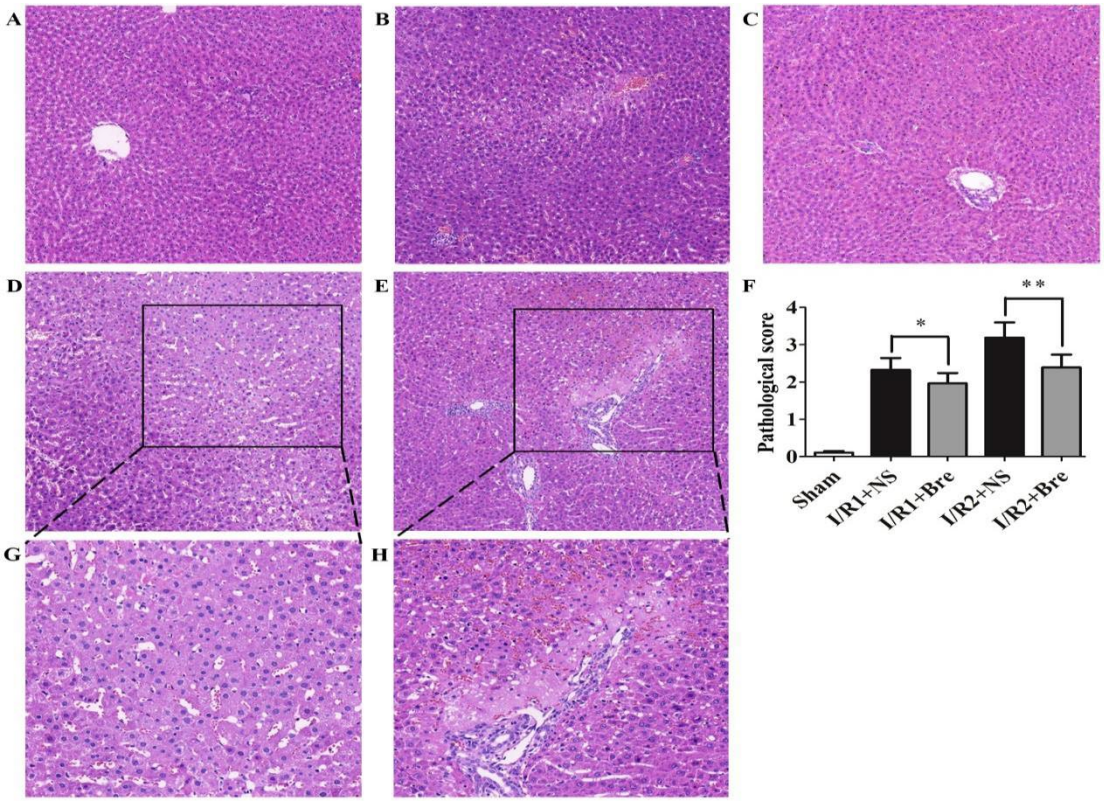

*Figure 2: Representative photomicrographs of the liver tissue sections from different groups after 6 h reperfusion. (A-E) H&E-stained histological images in the sham, I/R1 + NS, I/R1 + Bre, I/R2 + NS and I/R2 + Bre group, respectively (original magnification, x100). (G, H) Higher magnification pictures (x200). The sham group presented normal liver structure. The features of NS-treated groups were characterized by hepatocyte swelling, cytoplasmic vacuolation, unclear borders, neutrophil infiltration and even hepatocellular necrosis. All these changes were ameliorated by the administration of breviscapine. (F) Bar graph showed the pathological scores for the liver tissues. All data were showed in mean ± standard deviation. (n=8). *P<0.05, **P<0.01 vs. NS groups.*

Breviscapine treatment attenuates damage of liver tissue after I/R injury. To evaluate the protective effect of breviscapine on liver tissue, the damage scale was evaluated by histopathological examination under light microscope. As shown in Fig. 2, the sham group showed normal liver tissue structure. In

ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

contrast to the sham group, hepatocyte swelling, cytoplasmic vacuolation, unclear borders, and neutrophil infiltration were observed in the I/R1 + NS group, and these pathomorphological changes were more obvious as it prolonged to 60 min ischemia, showed in narrower hepatic sinusoids and hepatocellular necrosis. Compared to NS treatment, breviscapine groups displayed less prominent hepatocyte swelling, cytoplasmic vacuolation, neutrophil infiltration or hepatocellular necrosis, especially in 60 min ischemia group. All these could be specifically demonstrated by the evaluation of Suzuki's pathlogy score. As shown in Fig. 2F, the histologic injury score under breviscapine treatment was much lower than that in normal saline condition, especially in the I/R2 + Bre group ($P<0.01$). These findings suggest that breviscapine could decrease hepatic injury.

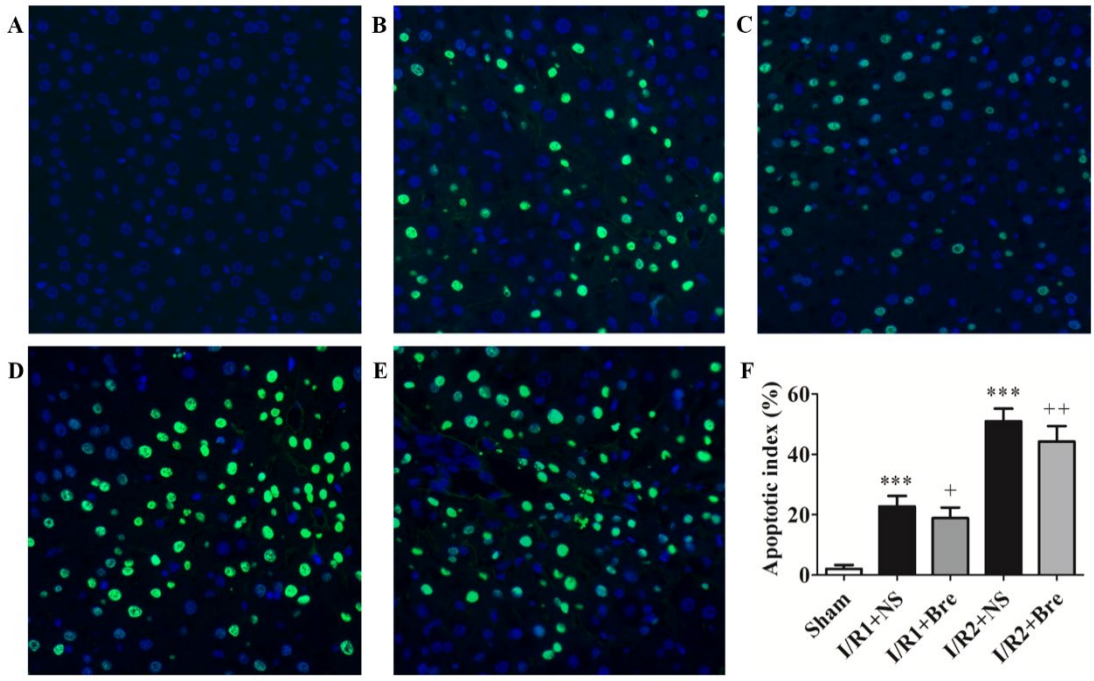

*Figure 3: Effects of breviscapine on hepatocyte apoptosis in rats. (A-E) The hepatocyte apoptosis in different groups was determined using TUNEL staining (original magnification, x400). The nuclei of TUNEL-positive hepatocytes were stained in green and those of normal cells were stained in blue. (F)The apoptotic index (AI) represents the proportion of TUNEL-positive cells to total number of hepatocytes. All data were showed in mean ± standard deviation. (n=8). ***P<0.001 vs. Sham group; +P<0.05, ++P<0.01 vs. NS groups.*

Breviscapine treatment declined apoptosis in the injury liver tissue after I/R injury. To determine the effect of breviscapine on hepatocyte apoptosis process in hepatic I/R injury, we used TUNEL assay to analyze the extent of hepatocyte apoptosis. As shown in Fig. 3A-E, almost no apoptotic cells in the sham group. However, the number of apoptotic cells were remarkably increased under I/R + NS condition, especially in the I/R2 + NS group. Notably, when rats were treated with breviscapine, fewer TUNEL-positive cells were seen, and the AI was markedly decreased compared with NS group, especially in the I/R2 + Bre group ($P<0.01$), seen in Fig. 3F. These indexes indicated that treatment with breviscapine could protect the liver against I/R induced-apoptosis.

Breviscapine increased the expression of GRP78 and Bcl2 while decreased the expression of cleaved caspase-3 and CHOP after I/R injury. In order to investigate the molecular mechanism underlying the reduction of ERS-induced apoptosis in response to breviscapine treatment, we examined the expression levels of GRP78, CHOP, Bcl2 and cleaved caspase-3, by western blotting. As shown in Fig. 4, in NS groups, a higher expression of GRP78, CHOP and cleaved caspase-3 were detected compared with sham group ($P<0.001$), along with a remarkable reduction of Bcl2 ($P<0.001$). Following breviscapine treatment, the expression of GRP78 and Bcl2 were increased, while the level of CHOP and cleaved caspase-3 were decreased compared with NS groups ($P<0.05$). These differential expressions suggest that breviscapine inhibits hepatocyte apoptosis might involve in ERS pathway.

ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

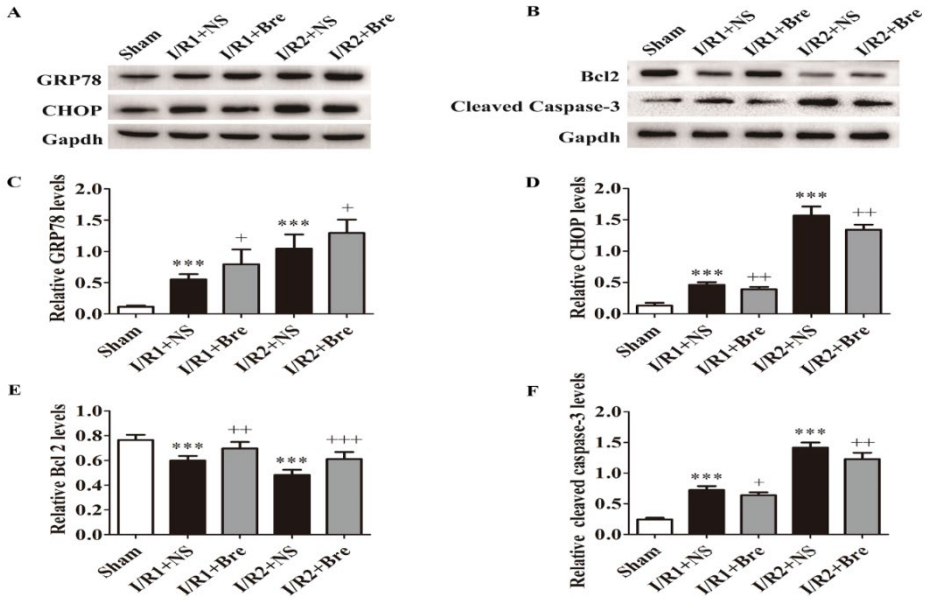

*Figure 4: Effects of breviscapine on the expression levels of GRP78, CHOP, Bcl2 and cleaved caspase-3. (A, B) Western blot images of the expression of GRP78, CHOP, Bcl2 and cleaved caspase-3 in different groups after 6 h reperfusion, respectively. (C-F) Bar charts showed the quantification of protein levels of GRP78, CHOP, Bcl2 and cleaved caspase-3, respectively. All data were showed in mean ± standard deviation. (n=8). ***P<0.001 vs. sham group; +P<0.05, ++P<0.01, +++P<0.001 vs. NS groups.*

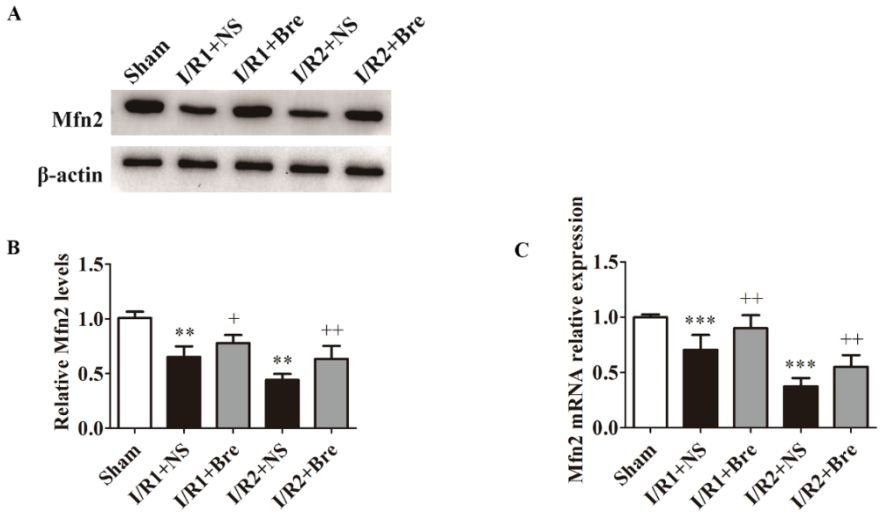

*Figure 5: Effects of breviscapine on the expression levels of Mfn2. (A) Representative western blot images of the expression of Mfn2 in different groups after 6 h reperfusion. (B) The bar chart showed quantification of the protein levels of Mfn2. (C) The mRNA levels of Mfn2 were measured by RT-qPCR analysis. All data were showed in mean ± standard deviation. (n=8). **P<0.01, ***P<0.001 vs, sham group; +P<0.05, ++P<0.01 vs. NS groups.*

Breviscapine upregulated the expression of Mfn2 during I/R injury. Mfn2, a mediator protein, distributes not only on the mitochondria membrane but also endoplasmic reticulum. Therefore, the expression of Mfn2 may be used to explore its potential protection mechanism of breviscapine. As shown in Fig. 5, the results of western blot analysis showed a decrease of Mfn2 level in NS groups compared with the sham group (P<0.01). Breviscapine treatment significantly upgraded the levels of Mfn2 than that in NS groups (P<0.05). Besides, concentration of Mfn2 mRNA, another index of expression of Mfn2, were detected by RT-qPCR for double testify. Analogously, the mRNA concentrations of Mfn2 in the NS groups also decreased compared with those in the sham groups (P<0.001). In Bre groups, the mRNA concentrations of Mfn2 were obviously raise compared with NS groups (P<0.01). These findings indicate that breviscapine could upregulates Mfn2 expression in hepatic I/R injury.

ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

4. Discussion

The question addressed by the present study was whether ERS-induced apoptosis is involved in the protective effect of breviscapine on hepatic I/R injury. Overall, the study demonstrated that injection of breviscapine could ameliorate hepatic I/R injury in rat model. The protective effect of breviscapine is supported by the results from decreased serum transaminases levels, ameliorated histological findings and inhibited subsequent apoptosis. Furthermore, we found that breviscapine altered the ERS pathway by upregulating GRP78 and downregulating CHOP in hepatic I/R injury. Meanwhile, breviscapine increased the expression of Mfn2 and Bcl2, leading to decreased the expression of cleaved caspase-3. Those results indicated that the protective effect of breviscapine may related to the inhibition of ERS-induced apoptosis as Mfn2 plays a vital role within this process.

Taking effective measures to ameliorate hepatic I/R injury is a long-term topic of liver surgery, which are critical to the success of surgery. Ischemic preconditioning (IPC) have been proved to alleviate hepatic I/R injury in clinical practice, but their limitations of prolonging surgery time and increasing surgical complications restrict their clinical applications [12,13]. In recent years, an effective pharmacological approach for ameliorating hepatic I/R injury is urgently needed, which is safer and more convenient.

Breviscapine has the effects of relaxing blood vessels, reducing vascular resistance, improving microcirculation, and inhibiting platelet aggregation [7]. Our previous study had elucidated the protective effects of breviscapine in hepatic I/R injury [10,14]. Additionally, the present study showed a decreasing of serum ALT and AST in treatment group of breviscapine compared with normal saline. In associated with the finding of histopathological changes, these data had even further demonstrated the protective effect of breviscapine on hepatic I/R injury.

However, the exact mechanisms of breviscapine protective effect on hepatic I/R injury are still obscure. Previous experimental evidence elucidates that ERS-induced apoptosis plays a critical role in hepatic I/R injury [3]. Unfolded protein response (UPR), an ERS-mediated self-protection mechanism, mainly triggered by three transmembrane receptor proteins which are inositol requiring enzyme 1 (IRE1), protein-kinase-RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6)[15-17]. Under normal physiological conditions, these three transmembrane proteins are bound to Glucose-regulated protein 78 (GRP78) which is regarded as an important molecular chaperone of endoplasmic reticulum and keep them inactive [18]. It was reported that GRP78 work as a catalyzer to facilitate protein folding by binding to those misfolded or unfolded proteins, while silencing GRP78 makes cells more susceptible to the ERS damage [19,20] .Many studies have also elucidated that the overexpression of GRP78 could moderate cells from stress [21,22]. When ERS occurs, GRP78 dissociates from the three transmembrane proteins mentioned above, triggers a downstream protein response, initiates the ERS pathway, and repairs damaged cells [23]. However, when ERS is severe or prolonged, it inevitably leads to apoptosis process in which the CHOP pathway is the main step [24]. Usually the expression level of CHOP is very low under normal conditions. Over-expression of CHOP leads to apoptosis process via inhibiting Bcl-2, result in activating the Caspase family [25,26].Caspase-3 is the executor of apoptosis and its activated form, cleaved caspase-3, plays a key role in the final procedure of apoptosis [27]. In present study, it was observed that a higher proportion of TUNEL-positive hepatocytes were detected in the normal saline-treated groups, accompanied with increasing of CHOP, GRP78 and cleaved caspase-3 expression and decreasing of Bcl-2 expression. In contrast, we found that breviscapine could effectively decreased the number of apoptotic cells, the expression levels of CHOP and cleaved caspase-3, while increased the expression levels of GRP78 and Bcl-2. These results indicated that ERS-induced apoptosis involves in the mechanism of hepatic I/R injury while breviscapine exert protective effects on hepatic I/R injury by inhibiting ERS-induced apoptosis.

To further elucidate the underlying mechanisms of breviscapine protective effect on hepatic I/R injury, Mfn2 is investigated in this study and it is recognized as a significant protein that regulates the endoplasmic reticulum-mitochondrial junctions and maintains its function [28]. Induction of ERS in mouse embryonic fibroblast (MEF) upregulated the expression of Mfn2, while the expression levels of other mitochondrial morphogen-related factors maintain steady [29]. However, induction of ERS in human airway smooth muscle (hASM) downregulated the Mfn2 expression [6] .As ERS relieved, Mfn2 expression would up-regulate and moderate mitochondrial morphology and dysfunction state [6]. It is suggested that Mfn2 may be associated with ERS. Moreover, there are many interface sites between the mitochondria and the endoplasmic reticulum with low expression of Mfn2, which can enhance the transport of Ca^{2+} from endoplasmic reticulum to mitochondria, making them more prone to apoptosis consequently [30].Therefore, we hypothesized that ERS-induced apoptosis is associated with the changes

ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

of Mfn2 and two of which are involved in the mechanism of hepatic I/R injury. In the present study, it was observed that the expression of Mfn2 in the NS-treated groups was significantly lower than that in the sham group, while the expression of Mfn2 in the Bre-treated groups were significantly higher than that in the NS-treated groups. These data suggested that breviscapine may exert a protective effect via upregulating the expression levels of Mfn2 during hepatic I/R injury.

In conclusion, the study demonstrates the phenomena that ERS-induced apoptosis results in hepatic I/R injury, and breviscapine has protective effect on hepatic I/R injury. The mechanism of this protective effect is likely due to upregulate the Mfn2 expression and inhibit the ERS-induced apoptosis, result in attenuating hepatic I/R injury. These findings suggest that breviscapine could be used as a potential pharmacological agent in liver surgery. Further studies are required to explore its pharmacological mechanisms and side effects.

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ISSN 2618-1584 Vol. 5, Issue 11: 1-9, DOI: 10.25236/FMSR.2023.051101

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